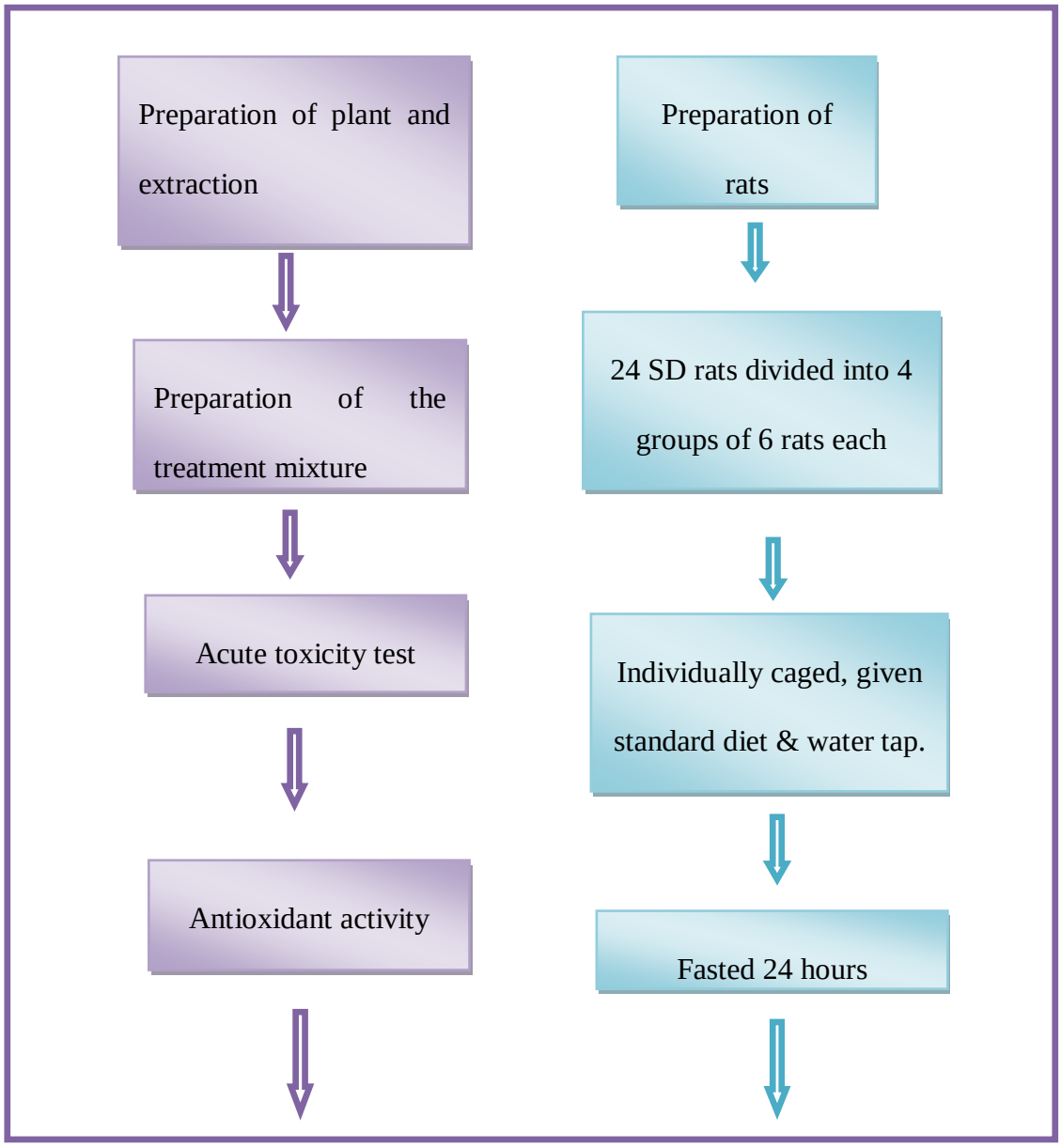
CHAPTER THREE

3.1. MATERIALS AND METHODS

3.1.1. Experimental Design



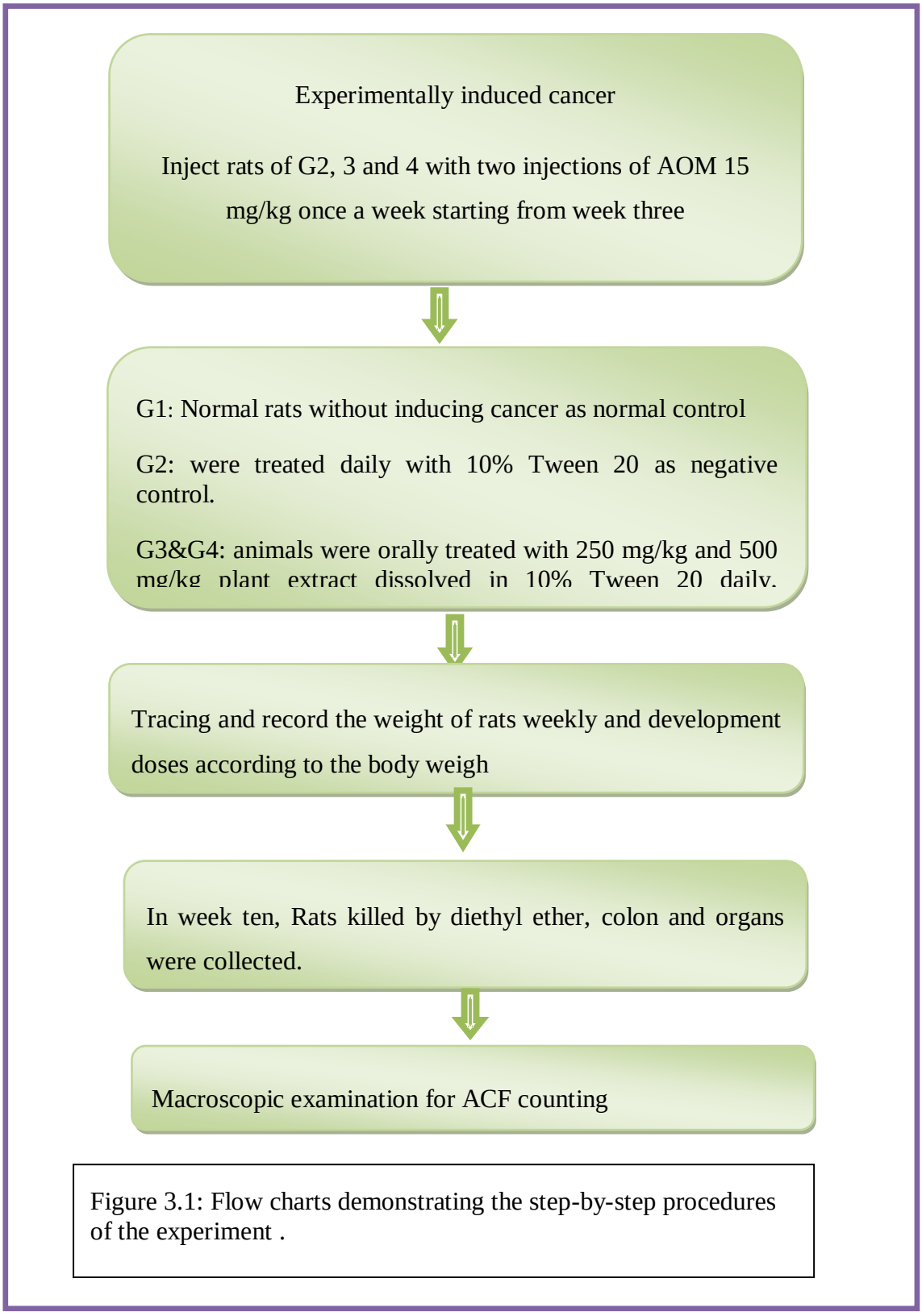


Figure 3.1: Flow charts demonstrating the step-by-step procedures of the experiment .

3.1.2. *P. niruri* Extract Preparation

P. niruri whole plant was obtained from Ethno Resources Sdn Bhd, Selangor Malaysia, and identified by comparison with the Voucher specimen deposited at the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur. The dried plant was finely powdered using electrical blender. 100 g of fine powder was soaked in 1000 ml of 95% ethanol in conical flask for 3 days in room temperature. After that the mixture was filtered using a fine muslin cloth followed by filter paper (Whatman No. 1) and distilled under reduced pressure in an Eyela rotary evaporator (Sigma-Aldrich, USA). The extract was placed in incubator to dry at 40^{o}C and the clear semisolid extract was dissolved by using the vehicle, 10% Tween 20.

3.1.3. Preparation of the Treatment Mixture

The clear semisolid extract was dissolved by using the vehicle, 10% Tween 20 (10 ml of absolute Tween 20 was added to 90 ml of distill water). 50 mg of semisolid extract was dissolved in 2 ml of the prepared solvent, and been given orally for rats (200 g) as a low dose, and 100 mg of the extract in 2 ml for 200 g rats as a high dose.

3.1.4. Acute Toxicity Studies

The acute toxic study was used to determine a safe dose for plant extract. Other thirty six *Sprague Dawley* healthy rats (18 males and 18 females), 8 weeks old and 180-200 g body weight was housed separately (one rat per cage) with 12 hours light/12 hours dark photoperiod and 50% to 60% humidity in order to maintain normal circadian rhythm in the animal room, were assigned equally into 3 groups labeled as vehicle (10% Tween 20), 2 g/kg and 5 g/kg plant extract in vehicle as low and high groups, respectively. The animals were fasted overnight (food but not water) prior dosing. Food was withheld for a further 3 to 4 hours after dosing (table 3.1.). The animals were observed for 30 min and 2, 4, 24 and 48 h after the administration for the onset of clinical or toxicological symptoms. Mortality, if any was observed over a period of 2 weeks. The animals were sacrificed on the 15^{th} day. Hematological, serum biochemical and histological (liver and kidney) parameters were determined following standard methods (Tietz et al., 1983). The handling of animals is accordance to the experimental protocols which were approved by the Committee for the Supervision of Animal Experimentation, in University of Malaya No. PM/28/08/2009/MAA (R).

Table 3.1 Acute Toxicity Study details.

species	Sprague Dawley rats
Age	6-8 weeks
Number of animals	6 rats -3 of each sex- per dose level
Dosage	Tow dose levels (2 g/kg and 5 g/kg body weight of plant extract preparation), plus a control group, exposures are single
Observation period	14 days

3.1.5. Antioxidant Activity

3.1.5.1. Ferric-reducing Antioxidant Power (FRAP) Assay

FRAP assay is a well known method and inexpensive procedure for measuring the ferric reducing ability (antioxidant power) levels in a sample. Reduction of ferric to ferrous ion reducing potential of the antioxidants to react with a ferrictripyridyltriazine (FeIII-TPTZ) complex and produce a colored ferrous tripyridyltriazine (FeII-TPTZ) form. The development of blue color complex which gives maximum absorption at 593nm (Benzie and Strain, 1996). This colour development indicates that a reductant (antioxidant) is present in the sample. Therefore, the FRAP assay is considered to be linearly related to the molar concentration of the antioxidants present. The assay was carried out at 37°C in a 96-well microtiter-plate. The assay mixture contained 10µl of the sample and 300µl of the FRAP reagent. FRAP values are obtained spectrophotometrically under kinetic mode using microplate reader for four minutes at 593nm. The

absorbance of each sample will be compared and calculated with the standard curve obtained from iron (II) sulphate–heptahydrate (FeSO4.7H2O.). The FRAP assay is considered to be linearly related to the molar concentration of the antioxidants present

3.1.5.2. DPPH Free Radical Scavenging Activity Test

The capacity of plant extract to remove 1,1-diphenyl-2-picryl-hydrazyl radical was determined by the method described by Shimada et al. (1992). Briefly, in 96-well plate, 1 ml of plant extract and 5 ml of freshly prepared 0.1 mM DPPH methanolic solution were thoroughly mixed and kept in the dark for 60 min. The absorbance of the reaction mixture at 517 nm was measured with a spectrophotometer. The blank was prepared by replacing the plant extract with methanol (1 ml). The percentage of free radical scavenging activity was calculated as follows:

Scavenging activity (%) = [1−(A517 nm, sample/A517 nm, blank)] × 100

3.1.6. Experimental Animals

Animal model which have used in this study are twenty four *Sprague Dawley* (SD) rats obtained from the Animal House, Faculty of Medicine, University of Malaya. Taking into account international principles and local regulations concerning the care and use of laboratory animals. The approximate weight of each rat was about 180-200 gram and approximate age upon received to animal room about 6-8 weeks. The animals been kept under controlled conditions

at room temperature (22-24℃), 50-60% humidity, 12 hr light-dark cycle and free access to standard diet and water ad labium. They have been acclimatized under standard laboratory condition for a period of two weeks before starting any experiment.

3.1.7. Chemoprevention of Plant Extract

3.1.7.1. Experiment Animals

Sprague Dawley adult male rats were divided randomly into 4 groups of 6 rats each. Each rat that weighted between 1800 - 200 g was housed separately (one rat per cage) (table 3.2.). The animals were maintained on standard pellet diet and tap water. Table 3.2. Animals experimental for Chemoprevention of plant extract.

Group	Subcutaneous Injection (S.C) 0.2 ml	Oral administration	Durations
1	Normal saline	Distilled water (Negative control)	2 months
2	Azoxymethane 15 mg/kg	10% Tween 20 (Cancer control)	2 months
3	Azoxymethane 15 mg/kg	*P. niruri* extract 250 mg/kg	2 months
4	Azoxymethane 15 mg/kg	*P. niruri* extract 500 mg/kg	2 months

3.1.7.2. Counting the ACF

ACF in the colon were counted as described by Bird (1987). Briefly, each colon was split open longitudinally and placed on a filter paper with the luminal surface open and exposed. Another filter paper was placed on top of the luminal

surface and fixed overnight using 10% buffered formalin. Each fixed colon was cut into proximal and distal portions of equal length and each portion was further cut into 2-cm long segments. Each segment was placed in a Petri dish and stained using 0.5% methylene blue solution for 5 min. The segments were transferred to another Petri dish containing buffer to remove excess stain, and then examined under a light microscope to score the total number of ACF as well as the number of crypts per focus figure. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from lamina to basal surface of cells and the easily discernible pericryptal zone.

Under light microscope, the total number of ACF as well as the number of crypts per focus was counted and the mean was founded to be compared in each group with the control group.